

# Continuous apple consumption induces oral tolerance in birch-pollen-associated apple allergy

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## Keywords

basophil activation test; birch pollen allergy; oral allergy syndrome; oral tolerance induction.

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## Abstract

**Background:** Patients with birch pollen allergy (major allergen: Bet v 1) have often an associated oral allergy syndrome (OAS) to apple, which contains the cross-reactive allergen Mal d 1. As successful birch pollen immunotherapy does not consistently improve apple related OAS symptoms, we evaluated whether regular apple consumption has an effect on OAS and immune parameters of Mal d 1 or Bet v 1 allergy.

**Methods:** A total of 40 patients with a clear history of birch pollen rhinoconjunctivitis and associated OAS to apple were included in an open, randomized, controlled clinical trial: 27 patients consumed daily defined amount of apple (1–128 g), doubling the amount every two to three weeks, while 13 patients remained untreated. Primary endpoint was the proportion of patients that achieved tolerance to at least 128 g of apple at the end of the study after 8 months. Exploratory endpoints were questionnaire about cross-reactive food and pollen allergy symptoms, conjunctival provocation test with birch pollen and Bet v 1, and *in vitro* tests (tIgE, sIgE, and IgG4 to Mal d 1 and Bet v 1; basophil activation test with both allergens).

**Results:** Seventeen of 27 patients in active group and none of 13 patients in control group ( $P = 0.0001$ ) could tolerate a whole apple after the intervention. However, differences in endpoints reflecting systemic immune reactivity did not reach statistical significance.

**Conclusion:** In patients with OAS to apple, tolerance can be safely induced with slowly, gradually increasing consumption of apple. However, the observation of a relapse after discounting of apple consumption and absence of immunologic changes suggest that induced tolerance is only transient.

More than 70% of birch-pollen-allergic patients suffer from an oral allergy syndrome (OAS) to fruits and/or nuts, especially to apple (1–3). This pollen fruit syndrome is based on a molecular similarity of their major allergens (all PR-10) leading to clinical cross-reactivity. One of the best studied examples is the cross-reactivity between Bet v 1 in birch pollen and Mal d 1 in apple (4). PR-10 proteins are rapidly released during the chewing process, but rather labile and quickly destroyed by digestive enzymes in the oral cavity. A large proportion of patients with OAS avoid or only eat processed fruits, which contain only negligible amounts of PR-10 proteins. As fresh fruits are considered important for well-balanced diet (5), many of these

patients seek help in getting rid of these mostly harmless, but annoying symptoms. There is beneficial effect of birch-pollen-specific subcutaneous immunotherapy on OAS to apple, with excellent improvement in some (1, 6–9), but limited effect in other studies (10–15). However, successful birch-pollen-specific sublingual immunotherapy only improved respiratory symptoms but showed no consistent effect on OAS (12, 14–16).

In this study, we investigated whether it is possible to induce local oral tolerance in these patients by daily oral intake of increasing amounts of apple allergens. Furthermore, we analyzed the systemic immune response by several *in vivo* and *in vitro* markers.

## Methods

### Study design

This randomized clinical trial study was an open, controlled parallel group comparison made at the Division of Allergology, Inselspital, University Hospital of Bern, from December 2009 to August 2010. The study started and concluded outside birch pollen season. Patients were randomized by an independent person, at a ratio 2 to 1, based on sex, age, dose of apple-tolerated, intradermal tests (IDT), and ratio of specific IgE to Bet v 1/total IgE into a control group (13 patients) and an active group (27 patients). All patients gave written informed consent before enrollment. The study was approved by the local ethical committee.

### Patients

Forty patients with birch pollen allergy and associated OAS to apple were included. Inclusion criteria were positive skin prick test to birch pollen, positive IDT with Bet v 1 and Mal d 1, sIgE to Bet v 1  $\geq 0.7$  kU/l, and positive oral provocation test (OPT) with fresh apple (threshold amount  $\leq 64$  g). Exclusion criteria were anaphylactic reactions to fruits and nuts, sIgE to Pru p 3  $\geq 0.35$  kU/l, severe asthma, and immunotherapy within last 3 years.

Patients were advised to omit antihistamines during the treatment and had only nasal corticosteroids for the treatment of their seasonal rhinoconjunctivitis. All patients were provided a first-aid kit consisting of 20 mg cetirizine and 100 mg prednisolone, to be taken in case of systemic IgE-mediated reactions (urticaria, asthma, and anaphylactic shock).

### Oral provocation test

Oral provocation test was carried out with a fresh Golden Delicious apple. We intentionally omitted blinded food challenge as the processing needed for the blinding may alter Mal d 1 content. The patients were given increasing amounts of apple which they chewed for 60 s in more chews. The dose was doubled every 5 min starting with 1 g up to 128 g. The OPT was stopped if relevant subjective symptoms (itching and loss of sensation) were reported or objective symptoms (swelling of lips and hoarseness) were observed, or the maximum test quantity of 128 g (double of maximally tolerated dose at T0) was tolerated.

### Intradermal tests

After verification that the patient has been off antihistamines and topical corticosteroids for at least 7 days, IDT were carried out with recombinant rBet v 1 and rMal d 1 (Biomay AG, Vienna, Austria) in four dilutions. The amounts of rBet v 1 were 0.00002, 0.0002, 0.002, and 0.02  $\mu$ g, and those of rMal d 1 were 0.0002, 0.002, 0.02, and 0.2  $\mu$ g. Reactions were documented after 15 min. The surface of the wheal was calculated according to the formula  $A = \left(\frac{D_1 + D_2}{2}\right)^2$ .  $D_1$  and  $D_2$  represent the mutual perpendicular diameters of the wheal.

### Conjunctival provocation tests

Conjunctival provocation tests (CPT) were carried out with Bet v 1 in concentrations 1.1, 3.3, and 10  $\mu$ g/ml and with birch pollen extract (Allergopharma, Reinbeck, Germany) in concentrations 550, 1650, and 5000 BE/ml. After demonstrating tolerance to the diluents alone, increasing amounts of birch pollen extract were dropped into conjunctival sac at 10-min intervals. Symptoms (itch, redness, weeping, and swelling of conjunctiva) were recorded on the symptom score scale.

### Oral tolerance induction protocol

Oral tolerance induction (OTI) consisted of two phases:

#### *Build-up phase*

Patients consumed daily small amounts of apple. Starting dose was the largest amount tolerated in the preceding OPT. The patients chewed the defined portion of apple carefully for about 1–2 min. The rest of the apple was stored for up to 2 days in the refrigerator as Mal d 1 content changes significantly after some weeks of storage (17). Oxidized (brownish) parts were not eaten, as their Mal d 1 content is low. The daily amount of apple was doubled every 2–3 weeks if the current amount was well tolerated.

#### *Maintenance phase*

When the whole apple (150–200 g) was tolerated without symptoms, the subject continued eating at least three apples/week to maintain tolerance.

### *In vitro* tests: total IgE, specific IgE and IgG4 to Bet v 1 and Mal d 1

Serum samples were taken from each subject before and at the end of study and stored at  $-20^\circ\text{C}$  until used for analysis. Serum total IgE and specific IgE and IgG4 antibodies to Bet v 1 and Mal d 1 were determined using the ImmunoCAP system (Phadia, Uppsala, Sweden) according to the manufacturer's instructions.

### Basophil activation test

Basophil activation test (BAT) was performed using reagents/protocols of Flow2CAST (Bühlmann, Schönenbuch, Switzerland) with slight modifications; 50  $\mu$ l EDTA blood from birch-pollen-allergic patients was stimulated with 50  $\mu$ l (0.5  $\mu$ g/ml) anti-IgE (clone E124.2.8, mouse IgG1k; Beckman Coulter, Marseille, France), 50  $\mu$ l fMLP, or 50  $\mu$ l (in six concentrations from 0.1 pg/ml to 10 ng/ml) recombinant rBet v 1 and rMal d 1 (both Biomay AG, Vienna, Austria) in 100  $\mu$ l stimulation buffer (without IL-3) for 30 min at  $37^\circ\text{C}$ , 5%  $\text{CO}_2$ .

Cells were stained simultaneously with anti-CCR3-APC and anti-CD63-FITC (BioLegend, San Diego, CA, USA). From the whole-cell population, a primary gating region was set around the monocyte/lymphocyte overlap region, where basophils are located. Basophils were selected on the basis of low granularity and their high expression of CCR3 (18). At

least 600 basophils were acquired using a FACSCanto® (BD Bioscience, San Jose, CA, USA), and basophil activation was expressed as percentage of CD63-positive basophils. Data were analyzed using FLOWJo software (TreeStar, Ashland, OR, USA). To determine basophil sensitivity, data from each visit were fitted into a sigmoid curve, and the  $\log_{10}$  concentration of allergen that results in 50% of maximal activation was determined as  $LC_{50}$  (19).

### Endpoints and statistical analysis

Primary endpoint was the proportion of patients that achieved tolerance of 128 g of apple in the active group vs the control group (Fisher's exact test). For the missing values, the last observed value was carried forward.

Secondary exploratory endpoints were clinical questionnaire about cross-tolerance to other PR-10 protein-containing fruits/nuts and a symptom diary concerning their pollen allergy symptoms, wheal size at IDT, and the symptom score at CPT. Laboratory exploratory endpoints were levels of tIgE, sIgE, and IgG4 to Mal d 1 and Bet v 1 and basophil reactivity to Mal d 1 and Bet v 1. For secondary exploratory endpoints,  $\Delta_{T0-T8}$  was calculated for each patient. The active group was compared with the control group. Significance was calculated using Mann-Whitney *U*-test.

## Results

### Demography, baseline values

Main demographic characteristics, allergy history, and test results at baseline (T0) are summarized in Table 1.

### Withdrawals from the study

Five of 27 patients in active group and none of 13 patients in control group withdrew. Two patients withdrew because of side-effects; two patients withdrew because of personal reasons. One patient forgot to eat apple while he was on holidays for 1 month during the study period. He had already

tolerated a whole apple daily, but after the 1 month break in OTI, OPT was again positive at 4 g.

### Primary endpoints

Tolerance of at least 128 g of apple (the highest amount at OPT) at T8 was achieved in 17 of 27 patients in active group and none of 13 patients in control group ( $P = 0.0001$ ).

The 17 responders reached the maintenance dose after an average time interval of 20 weeks (7–30 weeks) of OTI. They continued to eat on average 4–7 apples per week.

Five patients in the active group could not successfully reach maintenance dose despite repetitively trying eating apple. These patients were defined as nonresponders. At T8, they could tolerate slightly higher amounts of apple (Table 2).

Relevant side-effects occurred in two of 25 patients (two patients withdrew owing to personal reasons) in the active group and none of 13 patients in control group. One patient with severe birch pollen rhinoconjunctivitis experienced an increase in OAS symptoms (itching and hoarseness) to a previously tolerated apple dose during the birch pollen season, which prompted her to stop eating apple. Another patient reported diarrhea after 3 weeks of continuous apple exposure. Both patients withdrew. These reactions were not severe; first-aid kits were never used.

### Exploratory endpoints

The five of 27 patients from active group who withdrew were not included in exploratory endpoints analysis as we could not perform tests at T8. Exploratory endpoints did not show any significant difference neither between active and control group nor between responders and nonresponders.

### Cross-reactive food, symptoms of pollinosis.

Patients were also advised to try small amounts of cross-reactive fruits that were reported to cause OAS at T0. No formal OPT was carried out for these fruits. In active group, 29% of patients reported that after OTI to apple, they could

**Table 1** Demographic characteristics and allergy history of patients randomized in the study

	All	Active group	Control group
<i>N</i>	40	27	13
Age (years)	37 (18–61)	36 (18–61)	42 (24–61)
Mean (range)			
Men (%)	12 (30)	9 (33)	3 (23)
Women (%)	28 (70)	18 (67)	10 (77)
tIgE (kU/l)	127.5 (53.4–401.0)	147.5 (70.0–473.5)	103.8 (46.8–355.5)
Median (quartiles)			
sIgE Bet v 1 (kU/l)	15.85 (6.8–33.73)	14.30 (7.2–44.4)	17.60 (5.6–33.7)
Median (quartiles)			
sIgE Mal d 1 (kU/l)	3.56 (0.8–13.7)	3.56 (0.8–15.0)	5.21 (1.4–13.7)
Median (quartiles)			
Amount of apple (g) tolerated at OPT at T0	4 (1–64)	2 (1–64)	4 (1–32)
Median (range)			

**Table 2** Median (min–max) amount of apple (g), tolerated at oral provocation test (OPT) at T0 and T8 in active and control group and subgroup analysis of active group

	Active group			Control group
	Subgroup analysis of active group			
	Responder	Nonresponder	Dropout	
<i>N</i>	17	5	5	13
OPT T0	2 (1–32)	4 (1–64) g	4 (1–64)	4 (1–32) g
OPT T8	128 g	16 (1–64) g	Not done	4 (1–16) g
$\Delta_{T8-T0}$	126 (69–127) g	8 (0–60) g		0 (–24–6) g
<i>P</i>	0.0009			

tolerate pears, 27% cherries, 23% hazelnuts, 14% walnuts, and 18% peaches. One patient in control group reported the disappearance of OAS to peaches (14%) (Supporting information Table S1).

Not only 10 of 27 patients in active group reported improvement in seasonal rhinoconjunctival symptoms in symptom score in comparison with previous year but also did four of 13 patients in control group. In both birch pollen seasons, the pollen level was comparable. Neither the results of IDT nor the results of CPT with Mal d 1 and Bet v 1 revealed a significant change in  $\Delta_{T0-T8}$  in active vs control group (Supporting information Figure S1, S2).

### Laboratory endpoints

There was no significant change in  $\Delta_{T0-T8}$  in tIgE, sIgE, and IgG4 level to Mal d 1 and Bet v 1 in active vs control group. Results of BAT with Mal d 1 and Bet v 1 revealed no significant change in  $\Delta_{T0-T8}$  in basophil reactivity or sensitivity in active vs control group (Supporting information Figure S3, Table S2).

### Discussion

Former studies on oral apple-induced symptoms were performed with birch-pollen-specific immunotherapy. In this study, we used increasing oral doses of apple allergens to investigate whether it is possible to induce oral tolerance in patients with Bet v 1-associated OAS. Apple allergens were provided by increasing amounts of Golden Delicious apple, which have to be chewed for at least 1 min. Golden Delicious is an apple strain with a high content of Mal d 1 (17). However, PR-10 proteins are highly labile, and they are rapidly denaturated in the oral cavity. Therefore, the allergen exposure provided by the oral application of raw apple is subject to a high and unpredictable variability. Compared with SLIT in OTI, this variability is enhanced because of additional factors influencing the allergenic content of a Golden Delicious apple such as cultivar, place of growing, weather, and storage (17, 20, 21). However, a maintenance dose of our OTI treatment was approximately 620–1240  $\mu$ g (17) and markedly higher than a daily dose of a SLIT therapy (10–50  $\mu$ g Bet v 1) (22). Therefore, it can be assumed that the Mal d 1

exposure by OTI is at least as high as the Bet v 1 exposure provided by SLIT.

Our data show that 17 of 27 patients in the active group vs none of 13 patients in the control group could eat apple without OAS after ca. 20 weeks of apple consumption. We experienced that it was important to increase the dose slowly and gradually; actually, the dose was just doubled after a time interval of approximately 2–3 weeks. Under these conditions, no dangerous side-effects occurred and only two patients experienced local side-effects. We thus believe that OTI to apple at home is both safe and acceptable – as it is the case with conventional SLIT (23, 24).

Exploratory endpoints addressed clinical parameters (tolerance of other fruits, symptoms of rhinoconjunctivitis, CPT, and IDT), and extensive *in vitro* studies (specific IgE/IgG4 and BAT with both allergens) did not show any significant difference neither between active and control group nor between responders and nonresponders. This may be mainly due to the insufficient power of the study to detect small differences. However, very strong effects on cross-reactive fruits/nuts, rhinoconjunctivitis, sIgE, IgG4 levels, or basophil sensitivity are rather improbable. Also another group observed on two case reports that effect of OTI on other cross-reactive food is beneficial, but limited (25).

Five patients were not able to increase the tolerated dose of apple substantially: two of them increased the dose quite rapidly and continued to have symptoms and for three, the reason for the failure of OTI is unclear. Exploratory endpoints did not reveal a difference between responders and nonresponders regarding immune-modulatory markers.

One patient with initial successful OTI (tolerance of whole apple) forgot to eat apple for 1 month. Following this interruption, OPT was again positive at 4 g apple. A similar loss of apple tolerance after 2–4 weeks without apple consumption was already observed previously in the planning phase of this study (W. J. Pichler, personal observation). These observations suggest that the tolerance to apple after OTI is only a transient phenomenon. This transient nature of OTI would fit to the lack of an immunologic change observed on CPT and IDT tests as well as in the *in vitro* assays: It would also be in concordance with studies on OTI to cow's milk and hen's egg in children, where permanent tolerance could only be achieved in a small proportion of patients investigated (26). Although

there is *in vivo* and *in vitro* evidence that even heat-treated Mal d 1 can modify T-cell-mediated reactivity (27), we did not observe any systemic effect. However, the study was not powered and designed for the detection of such effects. Even in exploratory endpoints, we focused on IgE/IgG4, basophil responsiveness, and clinical challenge tests.

The transient nature of the observed effects may be explained by the concept of *tachyphylaxis* of IgE-mediated signals on mast cells (28). However, the persistence of allergic reactions to other cross-reactive fruits/nuts argues against this, as mast cells having undergone tachyphylaxis and having depleted their histamine pool should have lost reactivity to other allergens as well. From a mechanistic point of view, OTI appears to be similar to drug desensitization, which is also defined as induction of a state of *temporary* unresponsiveness to a drug responsible for a hypersensitivity reaction. But in contrast to OTI, drug desensitizations are achieved rapidly via oral or parenteral drug application. The mechanisms and molecular targets of drug desensitization are not well understood (29).

The protocol of our OTI is very similar to protocols of SLIT, which is assumed to induce a more stable tolerance and affects other organ systems such as the respiratory tract. A tolerance to grass or other orally applied allergens after a few weeks of treatment is indeed a well-known phenomenon. Thus, the high rate of responders in OTI regarding apple OAS is compatible to the experience with SLIT. On the one hand, the failure by OTI to induce a persistent effect on OAS as well as on immune parameters raises questions regarding the comparability of OAS vs SLIT and needs to be further investigated.

In conclusion, the strength of our study is its simplicity and clinical efficacy. The apple OTI is cheap and safe. All patients who could eat a whole apple without OAS were

highly satisfied with the result and continued to eat apple regularly even after completion of the study.

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## Conflict of interest

All authors declare no conflict of interest concerning the planning, conduction, and publication of this study.

## Supporting Information

Additional Supporting Information may be found in the online version of this article found at: <http://www.wileyonlinelibrary.com>.

**Figure S1.** Wheal size at IDT with different amounts of rMal d 1 and rBet v 1 at T0 and T8.

**Figure S2.** Symptom score at conjunctival provocation tests with different concentrations of Bet v 1 and birch pollen extract at T0 and T8.

**Figure S3.** Basophil activation ( $\Delta$  CD63%) with different of rMal d 1 and rBet v 1 at T0 and T8.

**Table S1.** Reported cross – reactive food that caused OAS at T0 and T8 and % of improvement.

**Table S2.** Serological data.

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